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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/JP96/03730 <b>(22) International Filing Date:</b> 20 December 1996 (20.12.96) <b>(30) Priority Data:</b> 7/340629 27 December 1995 (27.12.95) JP <b>(71) Applicant (for all designated States except US):</b> ROHTO PHARMACEUTICAL CO., LTD. [JP/JP]; 1-8-1, Tatsuminishi, Ikuno-ku, Osaka-shi, Osaka 544 (JP). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> TANAKA, Hitoshi [JP/JP]; 361-5 Kitahanauchi, Shinjo-cho, Kitakatsuragi-gun, Nara 639-21 (JP). KANAI, Atsushi [JP/JP]; 3-12-14, Komagome, Toshima-ku, Tokyo 170 (JP). TAKANO, Toshiyuki [JP/JP]; 2-1, Miya-cho, Kumagaya-shi, Saitama 360 (JP). KAWABA, Takako [JP/JP]; 1-1-2802, Katayama-cho, Suita-shi, Osaka 565 (JP). WADA, Masako [JP/JP]; Sun-house green 405, 4-40, Yoshida 2-chome, Higashiosaka-shi, Osaka 578 (JP). <b>(74) Agents:</b> AOYAMA, Tamotsu et al.; Aoyama & Partners, IMP Building, 3-7, Shiromi 1-chome, Chuo-ku, Osaka-shi, Osaka 540 (JP).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> PHARMACEUTICAL COMPOSITION CONTAINING TREHALOSE  <b>(57) Abstract</b>  A pharmaceutical composition for medical treatment in the field of ophthalmology, which comprises trehalose having protecting effect on cornea, especially corneal endothelial and epithelial cells is provided.		

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## DESCRIPTION

## PHARMACEUTICAL COMPOSITION CONTAINING TREHALOSE

## TECHNICAL FIELD

The present invention relates to a novel use of trehalose in medical treatment in the field of ophthalmology. More specifically, it relates to a pharmaceutical composition containing trehalose, which shows protecting effect on cornea and is used safely as an intraocular irrigating solution, eye drops, or eye ointment.

## BACKGROUND ART

As the cornea is an organ exposed to the outward, the protection thereof is very important to effect medical treatment in the field of ophthalmology successfully. The cornea consists of three layers, namely, epithelium, stroma and endothelium. The epithelium and endothelium play an important role serving as a barrier for the stroma.

The total corneal water content is kept to an appropriate amount by controlling the balance between passive influx and active excretion of intraocular water so as to maintain the normal function of cornea. Among cells consisting the cornea, endothelial cells play the most important role in which they control the balance between passive influx and active excretion of intraocular water through the pump-like function. As the corneal endothelial cells are responsible to the metabolism and transparency of

cornea, damage thereof possibly causes serious problems which could lead to the destruction of normal physiological function of cornea. For instance, the damage of endothelial cells can bring about imbalance of corneal water content, followed by corneal swelling and loss of transparency. The corneal endothelial cells, notwithstanding their significant role, have very low ability for cell division and are hardly repaired when damaged. This tendency is more marked in adult.

The number of intraocular surgery, such as cataract surgery, vitreous surgery or glaucoma surgery, has recently been increasing as the development of surgical techniques. Such intraocular surgery sometimes lasts for a long time, and throughout the operation, the operative site must be provided continuously with intraocular irrigating solution to keep the intraocular pressure normal, to prevent the exposed tissue from damage, and/or to wash away blood or tissue debris which might block the visual field. If the intraocular irrigating solution used has any unfavorable features, the continues contact therewith would damage corneal endothelial cells, which can adversely affect the postoperative recovery and be causative of corneal turbidity. Therefore, it is essential to use an intraocular irrigating solution capable of protecting the corneal endothelial cells.

In the early stage, an isotonic solution of a standard salt concentration, i.e., 0.9%, was widely used as intraocular irrigating solution. This solution, however,

proved to cause corneal swelling and damage endothelial cells. After then, it was suggested that a preparation consisting of ingredients similar to those of aqueous humor will be preferred. Accordingly, humor-like preparation  
5 such as BSS PLUS<sup>®</sup> (Alcon Laboratories) containing glutathione in the oxidized form or Opegurd MA<sup>®</sup> (SENJU PHARMACEUTICAL Co., LTD., TAKEDA CHEMICAL INDUSTRIES, LTD.), a salt-balanced solution is currently used. However, these existing preparations are not satisfactory  
10 with respect to the protecting effect on cornea.

The corneal epithelial cells exposed to outside are also readily damaged by various mechanical or non-mechanical factors, for instance, xerophthalmus (dry-eye), contact lens and the like. Dry-eye shows a tendency to  
15 increase in number and is one of serious problems. This disease is accompanied by equivocal complains of patients and therefore an effective treatment thereof is strongly demanded to improve the quality of patients' life. Further, as the progress of visual information-intensive  
20 society and the spread of contact lens, the diseases of the eyes are becoming more and more diversifying. In this respect, an effective protection of corneal epithelial cells exposed to the outward is one of the most important problems. To solve the problems, it is necessary to  
25 develop an eye drops or ointment capable of protecting the eye against foreign bodies or obstacles, thereby allowing corneal epithelial cells to function normally. An

artificial tear is sometimes used with a purpose of treating the disorder of corneal epithelial cells though, it does not show sufficient effect.

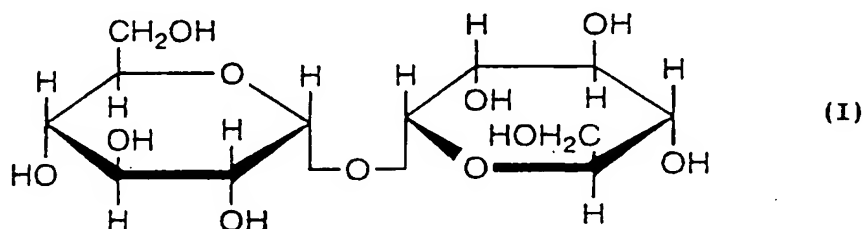
One of purposes of the present invention is to provide a pharmaceutical composition which shows sufficient protecting effect on ophthalmic tissue, especially cornea, and can be used as an intraocular irrigating solution, eye drops, or eye ointment safely. This purpose, however, could not be achieved easily because of specific physiological characteristics of ophthalmic tissue which are utterly different from others. Such differences can be attributed to the fact that the ophthalmic tissue is an extremely elaborate sense organ. Therefore, in the development of an intended pharmaceutical composition, many special problems had to be solved.

#### DISCLOSURE OF INVENTION

The present inventors extensively studied to find a substance useful as an active ingredient of a pharmaceutical composition for various medical treatments in the field of ophthalmology and have found that trehalose shows an excellent protecting effect on ophthalmic tissue. Trehalose is a disaccharide shown by the formula (I) below, which has long been known to be contained in many foods utilized by human beings, such as mushrooms, bread yeast, seaweeds, shrimps and the like. Prior to the present invention, it had been suggested that trehalose can be used as an ingredient of cosmetics, or conservative solutions for sperm or organ to be transplanted (Japanese Patent

Publication (KOKAI) No. 6-40801). However, no report has been presented showing or indicating that trehalose has cornea protecting activity and/or can be used as an active ingredient of a pharmaceutical composition in the field of ophthalmology.

Accordingly, the present invention provides a pharmaceutical composition for medical treatment in the field of ophthalmology, which comprises a compound of the formula (I):



#### BRIEF DESCRIPTION OF DRAWINGS

Fig. 1: A graph showing the protecting effect of the trehalose-containing composition of the present invention on corneal tissue.

Fig. 2: A graph showing the protecting effect of the trehalose-containing composition of the present invention on corneal epithelial cells.

Fig. 3: A graph showing the protecting effect of the trehalose-containing composition of the present invention on corneal endothelial cells.

#### BEST MODE FOR CARRYING OUT THE INVENTION

Examples of pharmaceutical compositions containing trehalose of the present invention include intraocular irrigating solution, eye drops and eye

ointment. As is seen from the experimental results below, the pharmaceutical composition containing trehalose shows an excellent protecting effect on cornea and is useful in the establishment of the purposes of the present invention.

5 For purposes of the present invention, the term "irrigating composition or irrigating solution" refers to a pharmaceutical composition of the present invention used to perfuse and wash ophthalmic tissue at various occasions. Therefore, throughout the specification, the term  
10 "irrigating composition or irrigating solution" is used in its broad sense including intraocular perfusate and eye wash.

Trehalose used as an active ingredient of the present composition can be obtained from a commercial  
15 source (e.g., Wako Junyaku, Co.) or prepared using a method known in the art or that described in literatures listed in MERCK INDEX, Vol. 11, pp. 1508, in the paragraph under "9496 Trehalose". Trehalose thus obtained may be free or in the form of hydrate. As is easily understood by one  
20 ordinary skilled in the art, both of hydrate and non-hydrate of trehalose are useful as an active ingredient of the pharmaceutical composition of the present invention. Further, in the pharmaceutical industry, a compound as an active ingredient is often converted into a derivative such  
25 as a pharmaceutically acceptable salt or ester to improve the solubility, safety or the like, which still has an in vivo activity equivalent to that of the free compound. Therefore, a pharmaceutical composition containing a



derivative of trehalose in the form of pharmaceutically acceptable salt, ester, or the like are fallen within the scope of the present invention, provided that the derivative has the cornea protecting activity equivalent to that of trehalose. Examples of such an ester include sulfuric ester and acetic ester. Sulfuric ester is known to show protective effect on skin (Japanese Patent Publication (KOKAI) No. 290808/1992). Accordingly, the sulfuric ester is expected to have an cornea protecting activity and be useful as an active ingredient of the pharmaceutical composition of the present invention. The sulfuric ester of trehalose can be prepared using a method known in the art, for example, in accordance with the method of Schweiger R. G. et al., Carbohydrate Research 21, 219-229 (1972).

When the pharmaceutical composition of the present invention is an intraocular irrigating solution or eye drops, it can be in the form of a solution, or a solid which is to be diluted with a solvent before use. In the latter case, an appropriate amount of the solid preparation of the present invention is dissolved, suspended or emulsified in a solvent such as distilled water, physiological saline or the like. Examples of solid preparations include tablet, granules, powders, which can be prepared in a conventional manner. The preparations are preferably sterilized by known method such as sterilizing filtration or heat sterilization.

The concentration of trehalose in the composition of the invention can be, in general, between about 0.01% and about 20% as a final concentration. Although preferred concentration varies from one preparation to another, it should be decided considering various factors such as corneal protecting effect, osmotic pressure, ion balance and the like. The protecting effect of the composition on the corneal epithelial and endothelial cells could be increased by adding more trehalose. For this purpose, the composition would preferably contain trehalose at a concentration of 0.1%, more preferably 0.3%, at least.

On the other hand, the composition of the present invention being applied to the ocular mucous membrane directly, the osmotic pressure thereof must be adjusted properly so as to ensure the safety and reduce the irritation. For this purpose, the concentration of salts in the composition must be decreased depending on the increase in the concentration of trehalose. This, however, would cause imbalance of ions in the composition. It is well known that the ion balance involving  $\text{Na}^+$  and  $\text{K}^+$  in the aqueous humor and tear is very important for the normal function of corneal cells. Therefore, the concentration of salts must be adjusted properly so as not to cause the imbalance of ions. From this viewpoint, the composition would preferably contain trehalose at a concentration of 5%, more preferably 3.5%, at most, in order to achieve the

protecting effect without affecting reversely the ion balance.

Accordingly, in general, the concentration of trehalose in the composition of the present invention can be between 0.01% - 10%, preferably 0.1% - 5%, more preferably 0.3% - 3.5% for an intraocular irrigating solution; and 0.01% - 20%, preferably 0.1% - 10%, more preferably 0.5% - 5% for eye drops or eye ointment.

The pH of the composition of the present invention used as intraocular irrigating solution, eye drops or eye ointment will be adjusted to a value between pH 6.5 and 8.0 using any of known methods. Further, the osmotic pressure of a preparation can be adjusted in a conventional manner so as to keep the osmotic pressure-ratio between 0.5 and 5, preferably 0.8 and 2, in the case of eye drops; and between 0.8 and 1.5, preferably 1.0 and 1.2, in the case of intraocular irrigating solution.

The pharmaceutical composition of the present invention can be prepared by adding trehalose or a derivative thereof at a concentration as defined above into a mixture of other ingredients conventionally used in the art, and adjusting pH and osmotic pressure, if necessary.

Further, the composition of the present invention may contain an appropriate amount of one or more pharmaceutically acceptable additives generally used in the art in individual preparation, unless the additives affect adversely the establishment of purposes of the present invention.

Examples of additives generally used in an intraocular irrigating solution include electrolytes such as calcium carbonate, magnesium chloride, magnesium sulfate, sodium acetate, sodium phosphate, potassium phosphate, sodium citrate, and sodium hydrogen carbonate; 5 monosaccharides such as glucose; peptides such as glutathione, and glutathione disulfide; antibiotics such as penicillin G.

10 Examples of additives generally used in eye drops include preservatives such as chlorobutanol, sodium dehydroacetate, benzalkonium chloride, cetylpyridinium chloride, phenetyl alcohol, methyl paraoxybenzoate, and benzethonium chloride; buffering agents such as borax, boric acid and potassium dihydrogen phosphate; 15 viscosity-increasing agents such as methylcellulose, hydroxyethylcellulose, carboxymethylcellulose, hydroxypropyl methylcellulose, polyvinyl alcohol, sodium carboxymethylcellulose and chondroitin sulfate; solubilizing agents such as polysorbate 80 and 20 polyoxyethylene hydrogenated castor oil 60; and stabilizers such as disodium edetate and sodium bisulfite.

When the composition of the present invention is an eye ointment, any conventional base such as ophthalmologic white petrolatum, propeto or plastibase may 25 be used. Additives such as liquid paraffin are also usable.

The dosage and administration mode of the intraocular irrigating composition of the present invention

can vary depending on various factors such as the age of a patient, operative method and the like. The usual dosage, however, would be about 20 - 500 ml for cataract surgery; about 50 - 4,000 ml for vitreous surgery; and about 20 - 50 ml for glaucoma surgery.

The dosage of the eye drops of the present invention also varies depending on the age, conditions and the like of a patient to be treated. The usual single dosage, however, is 1 - 2 drops and administered one to six times daily. For eye ointment, an appropriate amount is placed in the conjunctival sac one to two times daily.

The present invention is further described in the following Examples, but should not be construed to limiting the scope of the present invention.

Example 1 Intraocular Irrigating Composition

Trehalose (dihydrate)	3.5 g
Sodium chloride	0.375 g
Potassium chloride	0.0358 g
Calcium chloride (anhydride)	0.0133 g
Magnesium sulfate (anhydride)	0.0145 g
Sodium acetate (trihydrate)	0.0599 g
Sodium Citrate (anhydride)	0.0878 g
Sodium hydrogen carbonate (anhydride)	0.21 g
D-glucose (anhydride)	0.15 g
1N HCl	appropriate amount
<u>Sterile distilled water</u>	<u>appropriate amount</u>
Total	100 ml

A sterilized preparation is prepared using the ingredients above in a conventional manner to obtain an intraocular irrigating composition (pH 7.4; and osmotic pressure-ratio, 1.1).

5     Example 2   Intraocular Irrigating Composition

	Trehalose (dihydrate)	0.3 g
	Sodium chloride	0.636 g
	Potassium chloride	0.0358 g
	Calcium chloride (anhydride)	0.0133 g
10	Magnesium sulfate (anhydride)	0.0145 g
	Sodium acetate (trihydrate)	0.0599 g
	Sodium Citrate (anhydride)	0.0878 g
	Sodium hydrogen carbonate (anhydride)	0.21 g
	D-glucose (anhydride)	0.15 g
15	1N HCl	appropriate amount
	<u>Sterile distilled water</u>	<u>appropriate amount</u>
	Total	100 ml

20     A sterilized preparation is prepared using the ingredients above in a conventional manner to obtain an intraocular irrigating composition (pH 7.4; and osmotic pressure-ratio, 1.1).

Example 3   Intraocular Irrigating Composition

	Trehalose (dihydrate)	5 g
	Sodium chloride	0.375 g
25	Potassium chloride	0.0358 g
	Calcium chloride (anhydride)	0.0133 g
	Magnesium sulfate (anhydride)	0.0145 g
	Sodium acetate (trihydrate)	0.0599 g

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	Sodium Citrate (anhydride)	0.0878 g
	Sodium hydrogen carbonate (anhydride)	0.21 g
	D-glucose (anhydride)	0.15 g
	1N HCl	appropriate amount
5	<u>Sterile distilled water</u>	<u>appropriate amount</u>
	Total	100 ml

10 A sterilized preparation is prepared using the ingredients above in a conventional manner to obtain an intraocular irrigating composition (pH 7.4; and osmotic pressure-ratio, 1.2).

Example 4 Intraocular Irrigating Composition

	Trehalose (dihydrate)	10 g
	Sodium chloride	0.195 g
	Potassium chloride	0.0358 g
15	Calcium chloride (anhydride)	0.0133 g
	Magnesium sulfate (anhydride)	0.0145 g
	Sodium acetate (trihydrate)	0.0599 g
	Sodium Citrate (anhydride)	0.0878 g
	Sodium hydrogen carbonate (anhydride)	0.21 g
20	D-glucose (anhydride)	0.15 g
	1N HCl	appropriate amount
	<u>Sterile distilled water</u>	<u>appropriate amount</u>
	Total	100 ml

25 A sterilized preparation is prepared using the ingredients above in a conventional manner to obtain an intraocular irrigating composition (pH 7.4; and osmotic pressure-ratio, 1.5).

Example 5 Eye Drops

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	Trehalose (dihydrate)	3.5 g
	Sodium chloride	0.4 g
	Potassium chloride	0.15 g
	Sodium dihydrogen phosphate	0.2 g
5	Borax	0.16 g
	Benzalkonium chloride	0.004 g
	<u>Sterile distilled water</u>	<u>appropriate amount</u>
	Total	100 ml

10 A sterilized preparation is prepared using the ingredients above in a conventional manner to obtain an eye drops (pH 7.4; and osmotic pressure-ratio, 1.1).

Example 6 Eye Drops

	Trehalose (dihydrate)	0.3 g
	Sodium chloride	0.8 g
15	Potassium chloride	0.15 g
	Sodium dihydrogen phosphate	0.2 g
	Borax	0.16 g
	Benzalkonium chloride	0.004 g
	<u>Sterile distilled water</u>	<u>appropriate amount</u>
20	Total	100 ml

A sterilized preparation is prepared using the ingredients above in a conventional manner to obtain an eye drops (pH 7.4; and osmotic pressure-ratio, 1.1).

Example 7 Eye Drops

25	Trehalose (dihydrate)	0.5 g
	Sodium chloride	0.6 g
	Potassium chloride	0.15 g
	Sodium dihydrogen phosphate	0.2 g



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Borax	0.16 g
Benzalkonium chloride	0.004 g
<u>Sterile distilled water</u>	<u>appropriate amount</u>
Total	100 ml

5 A sterilized preparation is prepared using the ingredients above in a conventional manner to obtain an eye drops (pH 7.4; and osmotic pressure-ratio, 1.1).

Example 8 Eye Drops

10	Trehalose (dihydrate)	5.0 g
	Sodium chloride	0.3 g
	Potassium chloride	0.15 g
	Sodium dihydrogen phosphate	0.2 g
	Borax	0.16 g
	Benzalkonium chloride	0.004 g
15	<u>Sterile distilled water</u>	<u>appropriate amount</u>
	Total	100 ml

A sterilized preparation is prepared using the ingredients above in a conventional manner to obtain an eye drops (pH 7.4; and osmotic pressure-ratio, 1.1).

20 Example 9 Eye Drops

	Trehalose (dihydrate)	10 g
	Sodium chloride	0.3 g
	Potassium chloride	0.15 g
	Sodium dihydrogen phosphate	0.2 g
25	Borax	0.2 g
	Benzalkonium chloride	0.004 g
	<u>Sterile distilled water</u>	<u>appropriate amount</u>
	Total	100 ml

A sterilized preparation is prepared using the ingredients above in a conventional manner to obtain an eye drops (pH 7.4; and osmotic pressure-ratio, 1.5).

Example 10 Eye Drops

5	Trehalose (dihydrate)	20 g
	Sodium chloride	0.04 g
	Potassium chloride	0.02 g
	Sodium dihydrogen phosphate	0.03 g
	Borax	0.04 g
10	Benzalkonium chloride	0.004 g
	<u>Sterile distilled water</u>	<u>appropriate amount</u>
	Total	100 ml

A sterilized preparation is prepared using the ingredients above in a conventional manner to obtain an eye drops (pH 7.4; and osmotic pressure-ratio, 2.0).

Example 11 Intraocular Irrigating Composition

	Trehalose (sulfuric ester)	3.5 g
	Sodium chloride	0.375 g
	Potassium chloride	0.0358 g
20	Calcium chloride (anhydride)	0.0133 g
	Magnesium sulfate (anhydride)	0.0145 g
	Sodium acetate (trihydrate)	0.0599 g
	Sodium Citrate (anhydride)	0.0878 g
	Sodium hydrogen carbonate (anhydride)	0.21 g
25	D-glucose (anhydride)	0.15 g
	1N HCl	appropriate amount
	<u>Sterile distilled water</u>	<u>appropriate amount</u>
	Total	100 ml

A sterilized preparation is prepared using the ingredients above in a conventional manner to obtain an intraocular irrigating composition (pH 7.4; and osmotic pressure-ratio, 1.1).

5     Example 12   Eye Drops

	Trehalose (sulfuric ester)	5.0 g
	Sodium chloride	0.3 g
	Potassium chloride	0.15 g
	Sodium dihydrogen phosphate	0.2 g
10	Borax	0.16 g
	Benzalkonium chloride	0.004 g
	<u>Sterile distilled water</u>	<u>appropriate amount</u>
	Total	100 ml

15     A sterilized preparation is prepared using the ingredients above in a conventional manner to obtain an eye drops (pH 7.4; and osmotic pressure-ratio, 1.1).

Example 13     Eye Ointment

	Trehalose (dihydrate)	3.5 g
	Liquid paraffin	5 g
20	<u>Ophthalmologic white petrolatum</u>	<u>appropriate amount</u>
	Total	100 g

A sterilized preparation is prepared using the ingredients above in a conventional manner to obtain an eye ointment.

25     Experiment 1   Evaluation of Protecting Effect of Trehalose on Corneal Tissue

The protecting effect of trehalose on corneal tissue was evaluated comparatively using a composition with or without trehalose, and rabbit corneoscleral sections.

Compositions to be Tested

5           1. Trehalose (+) preparation; a composition containing trehalose which is prepared in a manner similar to that described in Example 1.

          2. Trehalose (-) preparation; a composition containing ingredients similar to the one described in 1.  
10       above except for trehalose.

Method

Four male Japanese white rabbits with normal eyes were used. After sacrifice of the animals, the eyeballs were enucleated and corneoscleral sections were prepared.

15       These sections were fixed to a perfusion chamber according to the method of Dikstein et al. (S. Dikstein and D.M. Maurice, The Metabolic Basis to the Fluid Pump in the Cornea, J. Physiol. 221: 29-41, 1972). Immediately, perfusion was started with a syringe pump under the  
20       conditions: flow rate, 1.6 ml/h and internal pressure of cornea, about 15 mm Hg. Specifically, one eye was perfused with the trehalose (+) preparation, and the other with trehalose (-) preparation for 8 hours at 37°C. After perfusion, scleral fragment was removed along the limbus  
25       and the wet weight of cornea alone was measured. After drying for 24 hours at 120°C, the dry weight was measured. The water content (%) was calculated according to the formula below:

Corneal water content =

$$[(\text{Wet weight}) - (\text{dry weight})]/(\text{dry weight})$$

### Results

The results are shown in Fig. 1. The water content in cornea after the 8-hour-perfusion with trehalose (+) preparation and trehalose (-) preparation is  $5.24 \pm 0.21$  (mean  $\pm$  standard error,  $n = 4$ ), and  $6.89 \pm 0.25$  (mean  $\pm$  standard error,  $n = 4$ ), respectively. The difference between the water content of cornea after perfusion with two different preparations is significant ( $p < 0.05$ , see, Fig. 1). These results show that the composition containing trehalose has an ability to protect corneal endothelial cells and can prevent cornea from swelling.

### Experiment 2 Evaluation of Protecting Effect of Trehalose on Corneal Epithelial Cells

The protecting effect of trehalose on corneal epithelial cells was evaluated by "Neutral red assay" using rabbit corneal epithelial cells damaged by benzalkonium chloride.

### Compositions to be Tested

1. Trehalose (+) solution; a solution containing 7% trehalose in a medium.

2. Trehalose (-) solution; a solution containing 0.6021 % salt in a medium.

### Method

This experiment was carried out by the use of a commercially available CORNE PACK kit (Kurabo, Co.) for

neutral red assay according to the instructions attached thereto.

Rabbit corneal epithelial cells were used.

Freezed primary cultured cells were inoculated into two 25  
5 cm<sup>2</sup> flasks at 4000 cells/cm<sup>2</sup> and incubated for 5 days under the conditions of 36.5°C, 5% CO<sub>2</sub> (secondary culture).

Then, 100 µl each of the secondary culture was inoculated into a 96-well multi-plate at 2500 cells/well and incubated for 3 days under the conditions of 37°C, 5% CO<sub>2</sub> (third

10 culture). To the cultured corneal epithelium cells was added 50 µl of benzalkonium chloride solution of different concentration, i.e., 25 x 10<sup>-6</sup>, 50 x 10<sup>-6</sup>, 75 x 10<sup>-6</sup>, and 10 x 10<sup>-5</sup> %, which was followed by the addition of 50 µl of a

15 trehalose (+) solution containing 7% trehalose, or a trehalose (-) solution containing 0.6021% salt. To wells used for untreated control experiments was added 100 µl of culture solution. The plate was then incubated for 2 days in an incubator at 36.5°C, 5% CO<sub>2</sub>.

20 After the addition of 100 µl of neutral red solution into each well, the plate was incubated for 3 hours at 36.5°C, 5% CO<sub>2</sub>. The supernatant was discarded.

Cells were fixed and washed in 200 µl of 1% formalin solution containing 1% calcium chloride for 1 minute, and the supernatant was discarded. After washing with water,  
25 neutral red was extracted from the cells with 100 µl of 50% ethanol containing 1% acetic acid for 20 minutes. The extract was subjected to the spectrophotometric measurement

at 540 nm corresponding to the absorbance of neutral red to evaluate the neutral red intake by cells.

The blank experiments were carried out in parallel using cultures obtained in the same manner as those for untreated control experiments except that neutral red was not added. The absorbance was corrected by subtracting the average absorbance of blanks. The ratio between the corrected absorbance for the test (trehalose (+) or trehalose (-)) group to that for the untreated control group was calculated according to the formula below to obtain the survival rate of cells.

$$\text{Survival rate of cells} = 100 \times (A)/(B)$$

A: average absorbance of all the test solutions

B: average absorbance of untreated control solutions

### Results

When benzalkonium chloride was added to the cultured corneal epithelial cells at different concentrations ranging from  $25 \times 10^{-6}$  to  $10 \times 10^{-5}$  %, the survival rate of cells decreased concentration dependently. The decrease, however, was apparently prevented at every concentration of benzalkonium chloride when trehalose was added simultaneously (see, Fig. 2). This indicates that trehalose has an ability to protect corneal epithelial cells.

### Experiment 3 Evaluation of Protecting Effect of Trehalose-containing Intraocular Irrigating Solution

The present experiment was carried out to evaluate the usefulness of trehalose as intraocular irrigating solution using the trehalose (+) composition and, as a control, the trehalose (-) composition similar to those used in the Experiment 1 above.

#### Method

Six male Japanese white rabbits were subjected to general anesthesia and an incision of 3 mm wide was made in the sclera at 2 mm apart from the limbus, from which an ultrasonic tip attached to a phacoemulsifier-aspirator for cataract surgery (Haag Streit AG) was inserted. The anterior chamber was then perfused for 60 minutes at room temperature with 600 ml in total of trehalose (+) composition (test group) or trehalose (-) composition (control group). The thickness of the cornea was measured immediately, and on 1, 2 and 7 days after the perfusion with a ultrasonic pachymeter (MENTOR O & O).

#### Results

The results are shown in Fig. 3. In the figure, the "0" on the axis means that the measurement was performed immediately after the perfusion. The corneal thickness on the day 1 after perfusion was  $0.322 \pm 0.005$  mm (mean  $\pm$  standard error,  $n=3$ ) in the test group, and  $0.349 \pm 0.001$  mm (mean  $\pm$  standard error,  $n=3$ ) in the control group. The difference is significant ( $p<0.01$ ) (see, Fig. 3).

These results show that the composition containing trehalose has an ability to protect corneal



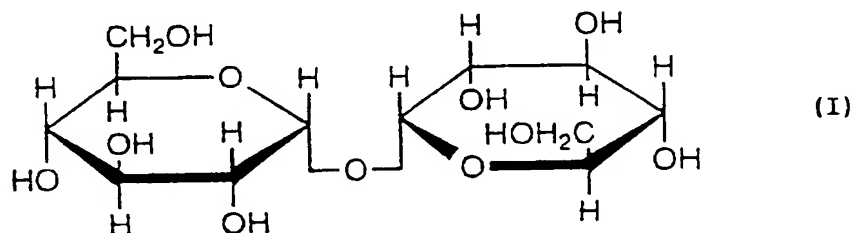
endothelial cells and thereby preventing cornea from swelling due to the irrigating solution.

#### INDUSTRIAL APPLICABILITY

As is evident from the results of experiments  
5 above, the composition of the present invention has an  
outstanding ability to protect ophthalmic tissues,  
especially corneal endothelial cells which are readily  
damaged during intraocular surgery. Therefore, the  
composition of the present invention showing improved  
10 safety is useful as an intraocular irrigating solution for  
intraocular surgery. Further, owing to the protecting  
effect of trehalose on corneal epithelial cells, the  
composition of the present invention is useful as an eye  
drops or ointment in the treatment of damaged cornea.

## CLAIMS

1. A pharmaceutical composition for medical treatment in the field of ophthalmology, which comprises a compound of the formula (I):



2. The pharmaceutical composition of claim 1, which is an eye drops.

3. The pharmaceutical composition of claim 1, which is an eye ointment.

4. The pharmaceutical composition of claim 2 or 3, which contains the compound (I) at a concentration between 0.01% and 20%.

5. The pharmaceutical composition of claim 1, which is an intraocular irrigating composition.

6. The pharmaceutical composition of claim 5, which contains the compound (I) at a concentration between 0.01% and 10%.

Fig. 1

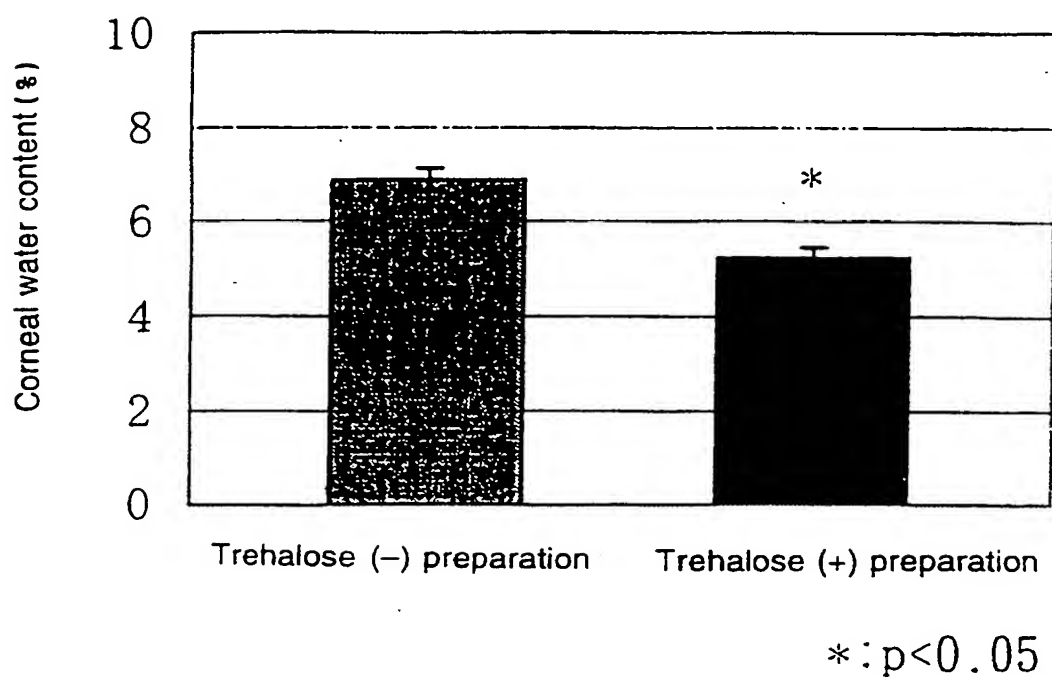
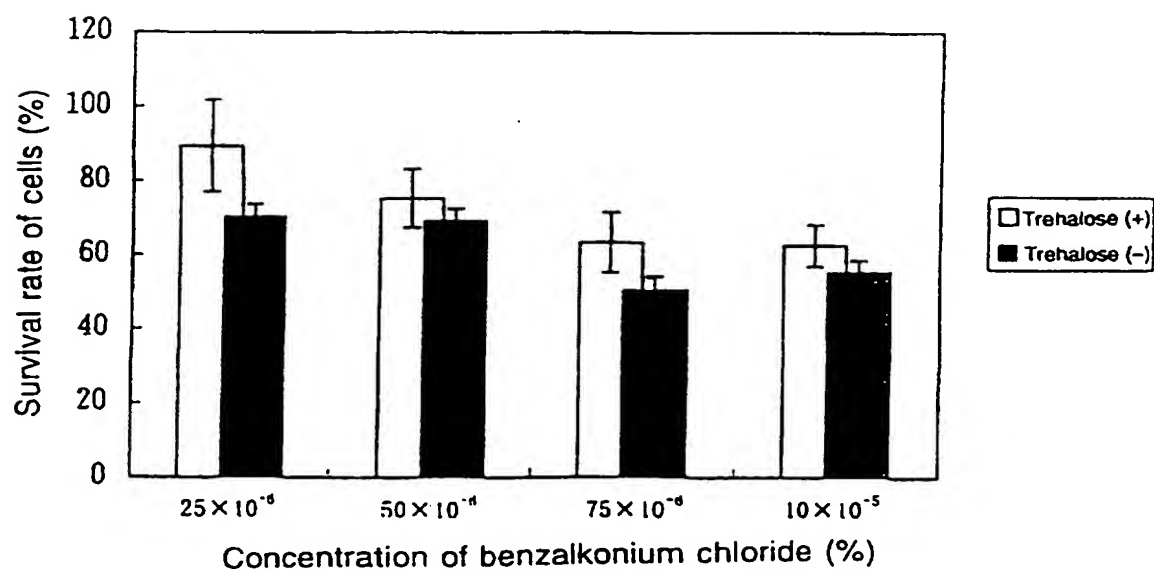
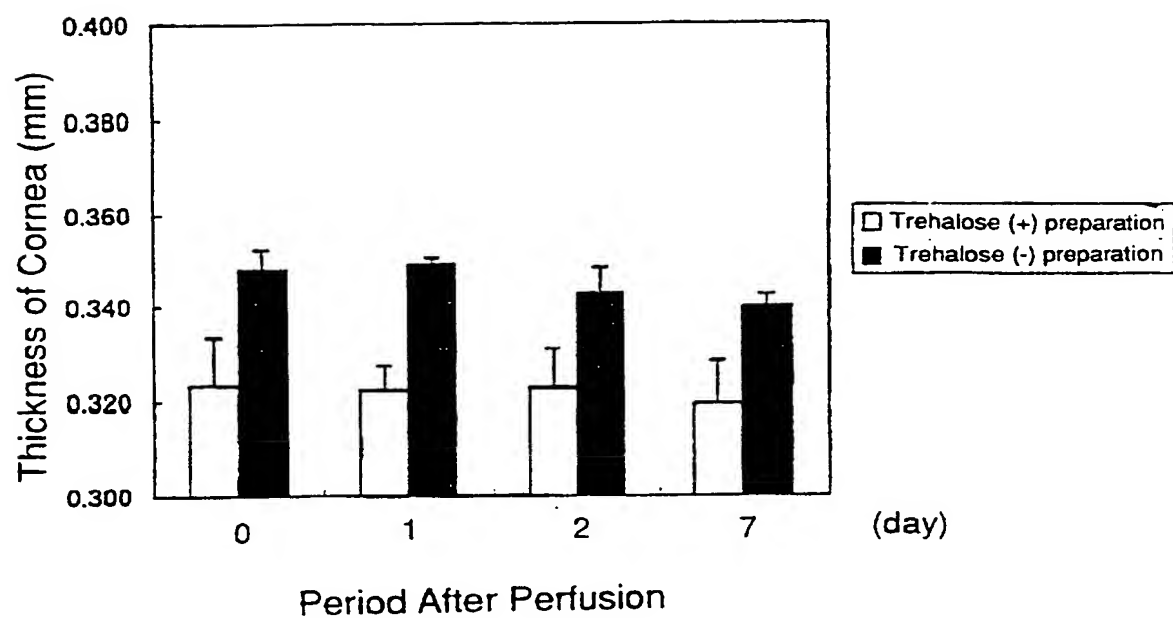


Fig. 2



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Fig. 3



# INTERNATIONAL SEARCH REPORT

Int. Application No  
PCT/JP 96/03730

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INVEST.OPHTHALMOL.VISUAL SCI., vol. 27, no. 3suppl, 1986, page 278 XP000645245 J.V.GREINER ET AL.: "TREHALOSE MAINTENANCE OF THE METABOLIC HEALTH OF THE CRYSTALLINE LENS DURING SEVERE TEMPERATURE STRESS" see abstract ---	1-6
X	DATABASE WPI Derwent Publications Ltd., London, GB; AN 94-329954 XP002027389 & JP 06 256 219 A (HISAMITSU PHARM CO LTD) , 13 September 1994 see abstract --- -/-	1-6

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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Date of the actual completion of the international search

12 March 1997

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# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/JP 96/03730

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE WPI Derwent Publications Ltd., London, GB; AN 89-215498 XP002027390 &amp; JP 01 151 528 A (TAIHO PHARM KK) , 14 June 1989 see abstract -----</p>	1-6